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ACUTE PNEUMOCYTE INJURY, POLY(ADP-RIBOSE) POLYMERASE ACTIVITY, AND PYRIDINE NUCLEOTIDE LEVELS AFTER *IN VITRO* EXPOSURE OF MURINE LUNG SLICES TO CYCLOPHOSPHAMIDE

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Abstract—Cyclophosphamide (CYC) is a metabolically activated, DNA-alkylating, antitumor agent that causes pulmonary fibrosis. BALB/cN (B) mice are sensitive and C57Bl/6N (C) mice are resistant to CYC-induced fibrosis. Pulmonary bioactivation may contribute to strain sensitivity. Therefore, we tested the intrinsic susceptibility of murine lung slices to cell injury by direct exposure to CYC for 2–8 hr. Injury was measured by release of lactate dehydrogenase (LDH). DNA damage activates the nuclear enzyme poly(ADP-ribose) polymerase (PAP, EC 2.4.2.30), causing depletion of its substrate, NAD. NAD can also be decreased by phosphorylation to NADP, as seen with oxidative stress. Depletion of NAD can lead to loss of ATP. Thus, we measured LDH release, PAP activation, NAD, NADP and ATP in slices incubated with or without the PAP-inhibitor, 3-aminobenzamide (3-AB). CYC (0.1 to 1.0 mg/mL for 4–8 hr) caused LDH release in slices from both murine strains, but LDH release was significantly greater in B lung slices than in C slices. After an 8-hr incubation 63.9 ± 3.7% (mean ± SEM) of total LDH was released from B lung slices with 1.0 mg CYC/mL, whereas only 45.8 ± 2.6% was released from C lung slices ($P < 0.05$). 3-AB reduced LDH release to 44.7 ± 2.4% in B slices and 28.1 ± 2.0% in C slices ($P < 0.05$ vs CYC only). PAP activity in nuclei isolated from CYC-treated B lung slices was increased 2- to 4-fold after 2 hr of incubation with 0.5 and 1.0 mg CYC/mL. PAP activation was delayed and reduced with incubation in 3-AB. PAP was activated 2-fold in nuclei from C slices treated with 0.5 mg CYC/mL for 2 hr. NAD was decreased at 2 and 4 hr in B slices treated with 0.5 and 1.0 mg CYC/mL, and at 4 hr with 0.1 mg CYC/mL. NAD depletion occurred only at 4 hr in the resistant C slices treated with 1.0 mg CYC/mL. CYC increased NADP by a similar extent in B and C lung slices. In B slices, NAD losses were approximately 4 times the increases in NADP. CYC did not decrease ATP in B slices and ATP dropped 25% only after 4 hr in the resistant C slices. We conclude that CYC is directly toxic to lung tissue and observe that strain sensitivity *in vitro* mirrors the sensitivity to fibrosis *in vivo*. PAP activation and oxidative stress may contribute to this toxicity.

Key words: cyclophosphamide; lung; poly(ADP-ribose) polymerase; NAD; ATP; mice

Pulmonary fibrosis is a chronic, life-threatening disease caused by many environmental contaminants, ionizing radiation and genotoxic drugs, such as CYC† [1–3]. We have studied the responses of murine strains that are sensitive or resistant to the DNA-damaging, antitumor agents, BLM and CYC, to elucidate critical events in the pathogenesis of pulmonary fibrosis [4–7]. C57Bl/6N (C) mice are sensitive to BLM and resistant to CYC, while an inverse relationship exists for BALB/cN (B) mice. After 7 days, CYC and BLM cause increases in mRNA encoding extracellular matrix components and transforming growth factor-β in the sensitive murine strain [6–8]. This stereotypic, delayed response to both agents suggests that murine strain variation is due to differential sensitivity to more acute actions of these drugs. Acute injury presumably leads to inflammation and direct and/or indirect effects on mRNA and protein levels.

Genotoxic fibrogenic agents, such as BLM and CYC, cause acute injury to pneumocytes, particularly endothelial and epithelial cells [1]. We found that murine sensitivity to direct cytotoxic effects of BLM in isolated lung slices correlates well with the *in vivo* sensitivity to BLM-induced pulmonary fibrosis [9]. These results support the role of acute cell injury in initiating BLM-induced fibrosis and indicate that murine lungs are intrinsically sensitive or resistant to BLM. It is not known whether CYC can injure lung tissue *in vitro*.

CYC is bioactivated through arachidonic acid cooxidation in lung tissue [10], and liver can metabolize CYC, yielding toxic metabolites [11]. Smith and Kehrer [10] demonstrated that cooxidation is greater in lung than liver, that cooxidation inhibitors reduce the lung toxicity of CYC, and that inhibitors of mixed-function oxidase do not protect lung from CYC. Although the nature and pulmonary effects of lung-specific metabolites of CYC have not been fully characterized, pulmonary cooxidation appears necessary for the pneumotoxic actions of CYC [10, 12].

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† Abbreviations: B, BALB/cN; C, C57Bl/6N; BLM, bleomycin; CYC, cyclophosphamide; LDH, lactate dehydrogenase; PAP, poly(ADP-ribose) polymerase; TCA, trichloroacetic acid; and 3-AB, 3-aminobenzamide.

Metabolic activation of CYC may yield species that cause oxidative stress [11, 13, 14]. Oxidative stress and/or CYC metabolites may cause DNA damage [15, 16].

DNA strand breaks of various types activate the nuclear enzyme PAP (EC 2.4.2.30) and cause depletion of its substrate, NAD [17, 18]. PAP activation and NAD depletion correlate with the sensitivity of murine lung slices to BLM. In addition, the PAP inhibitor 3-AB reduces PAP activation, NAD depletion and LDH release in BLM-sensitive lung slices [9, 19].

Oxidative injury may also cause NAD depletion by phosphorylation to NADP [20–22]. An NAD kinase may be activated by oxidative stress induced by quinones and peroxides. NAD depletion by this path appears to be 3-AB insensitive [21, 22].

NAD or ATP depletion may contribute to lethal cell injury, depending on the agent and cell types affected [19, 23, 24]. Our goals in the present study were to assess the possibility for pneumocyte injury and strain variation in mouse lung slices exposed to CYC, and to determine whether injury is 3-AB sensitive and related to changes in PAP, ATP, NAD, or NADP levels.

MATERIALS AND METHODS

Lung slices and treatments. Female C or B mice (Charles River Laboratories, Kingston, NY) were maintained on a 12-hr light/dark cycle with food and water *ad lib*. Lung slices were prepared as described previously [9]. For each time point in our experiments, three mice of each strain were anesthetized with sodium pentobarbital (100 mg/kg, i.p.). The thoracic cavity was opened, the dorsal aorta was cut, and the lungs were perfused free of blood with phosphate-buffered saline. The lungs were removed, the lobes were separated, and slices were cut (<1 mm) with a scalpel on a glass plate.

Slices were equilibrated by three washes ×5 min each at room temperature in Krebs–Henseleit buffer (pH 7.2) with 4.5 g glucose (Sigma Chemical Co., St. Louis, MO) per liter (Krebs/glucose buffer). Three to four slices (10–15 mg tissue) were placed in wells of 24-place culture plates in 1.0 mL Krebs/glucose and shaken [25]. The samples were preincubated at 37° for 15 min in 1.0 mL buffer with or without 2.5 mM 3-AB (Sigma) to inhibit PAP.

Initial studies indicated that exposure of slices to CYC (Sigma) for 2 hr was not sufficient to elicit toxicity, but that continuous exposure was effective by 4 hr (see Results). Therefore, lung slices were treated in triplicate with 0, 0.001, 0.01, 0.1, 0.5 or 1.0 mg CYC per mL Krebs/glucose buffer (1.0 mL) with or without 2.5 mM 3-AB for the entire incubation.

LDH assay. Samples of medium were taken at 2, 4 and 8 hr of incubation, and cell injury was monitored by LDH release into the buffer as described previously [9]. Tissue LDH was measured by homogenizing the slices in 0.25% Triton-X-100 (Kodak, Rochester, NY). The homogenate was centrifuged for 5 min at 15,000 *g*, and an aliquot of supernatant was taken for assay. LDH activity was measured in each sample by the linear rate of decrease in A_{340} of 0.19 mM NADH (Sigma) in the presence of 1.9 mM pyruvate (Boehringer Mannheim, Indianapolis, IN) [26]. Cumulative release of LDH activity was calculated and normalized to the total activity content of each sample.

PAP assay. Activity of PAP in nuclei isolated from lung slices was measured as described previously [9]. Nuclei were isolated from lung slices at 2 and 4 hr after initiating treatment. Slices were washed three times in Krebs/glucose buffer, and nuclei were isolated by homogenizing slices (10 strokes at 4°) with a Teflon pestle in lysis buffer containing 0.14 M NaCl, 1.5 mM $MgCl_2$, 10 mM Tris, pH 8.6, 0.5% NP-40. Aliquots were also taken for protein determination [27]. The homogenate was layered over 300 μL of lysis buffer containing 24% sucrose and 1% NP-40 (Sigma) [28]. The sample was centrifuged at 10,000 *g* × 15 min at 4°. The supernatant was discarded, and nuclei were washed and resuspended in 225 μL of reaction buffer (40 mM HEPES, pH 7.8, 130 mM KCl, 2.5 mM dithiothreitol, 225 mM sucrose, 2 mM EGTA, 2.3 mM $MgCl_2$). Reactions were initiated by the addition of 25 μL of 9.1 μM [U-^{14}C-adenine]NAD, 50 Ci/mol (Amersham, Arlington Heights, IL). The reactions were stopped after 15 min by the addition of 1.0 mL of ice-cold 12.5% TCA (Sigma). The samples were precipitated on ice for at least 2 hr and filtered on Whatman GF/C filters (Whatman, Maidstone, U.K.). Filters were washed three times with cold 10% TCA, once with acetone, dried, and counted by liquid scintillation in ScintiVerse BD (Fisher Scientific, Pittsburgh, PA). Net molar incorporation of [^{14}C]ADP-ribose was calculated based on the specific activity of ^{14}C in the substrate and a counting efficiency of 0.84.

Extraction of nucleotides. At appropriate times, slices were homogenized in 220 μL phosphate-buffered saline at 4°. An aliquot (20 μL) was taken for protein assay [27]. Protein in the remaining sample was precipitated with 10% TCA, and the supernatant was neutralized as described previously [19]. NAD, ATP and NADP were determined in all of the samples. Recovery of standard nucleotides that were added to samples of lung slices prior to homogenization was >94% in each case.

HPLC determination of NAD and ATP. Sample and standard nucleotides (Sigma) were measured by reverse-phase HPLC by the method of Jones [29], using a Waters U6K injector, two Waters 501 HPLC pumps, a Whatman Partisil PXS C8 column (25 cm × 4.6 mm) and a Waters 994 Programmable Photodiode Array Detector as described previously [19].

HPLC determination of NADP. Strong anion exchange HPLC was used to determine NADP [30]. The system described above was used with a HI-CHROM S5 SAX column (25 cm × 4.6 mm, Regis Chemical Co., Morton Grove, IL). The mobile phase was 0.1 M potassium phosphate (pH 5.5) pumped at 2.0 mL/min. Prior to analysis, 50 μL of sample was diluted to 200 μL with mobile phase containing 5.5 μM pyridoxine, and 200 μL was injected. A_{254} was recorded for nucleotides, and A_{334} was monitored for pyridoxine as the internal

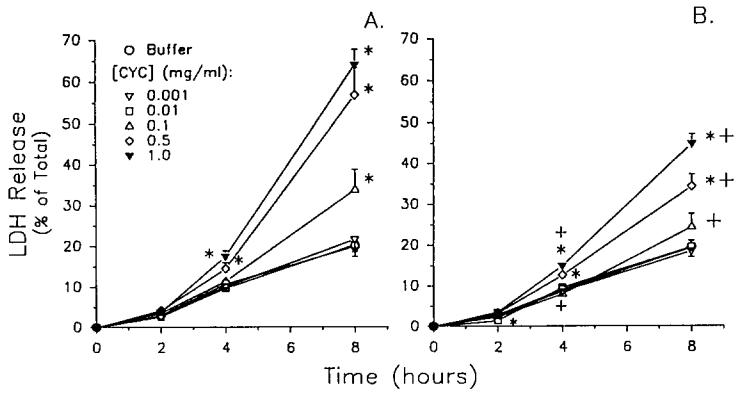

Fig. 1. Effect of CYC on LDH release from BALB/cN mouse lung slices in the absence (A) and presence (B) of 2.5 mM 3-AB. Data are presented as means ± SEM (N = 3). Key: (*) $P < 0.05$ for comparison with buffer control, and (+) $P < 0.05$ for comparison between slices incubated in the presence and absence of 2.5 mM 3-AB.

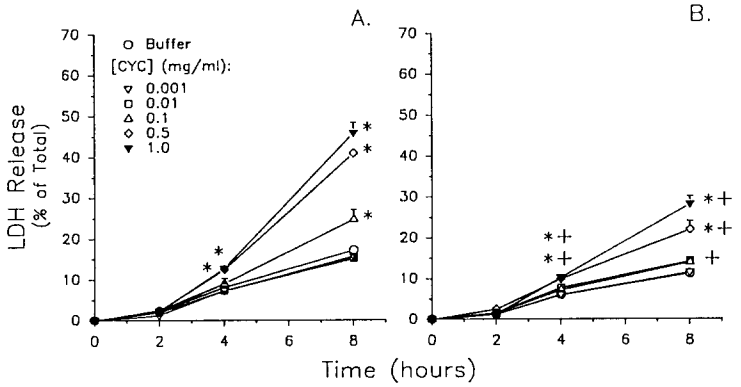

Fig. 2. Effect of CYC on LDH release of C57Bl/6N mouse lung slices in the absence (A) and presence (B) of 2.5 mM 3-AB. Data are presented as means ± SEM (N = 3). Key: (*) $P < 0.05$ for comparison with buffer control, and (+) $P < 0.05$ for comparison between slices incubated in the presence and absence of 2.5 mM 3-AB.

standard. The photodiode array detector was also used to determine UV spectra of eluted material, confirming the identity of adenine-containing nucleotides.

Statistics. ANOVA was used to assess significant differences at $P < 0.05$. ANOVA with contrasting was used for multiple comparisons [31].

RESULTS

LDH release. After 8 hr of incubation, control LDH release was (mean ± SEM, % of total) 19.9 ± 2.6% in B lung slices and 17.2 ± 2.5% in C slices (Figs. 1A and 2A). This basal release was similar to that observed in our previous study [9]. In preliminary studies we found that a 2-hr exposure to CYC was insufficient to cause LDH release in lung slices from either murine strain within the 8-hr incubation (data not shown). Bioactivation or sufficient exposure of critical sites might not be achieved within this time. Continuous CYC exposure, however, was toxic to lung slices, and significant LDH release was evident at 4 hr (Figs. 1 and 2, panels A). A concentration-related increase in LDH release was seen in slices from both murine strains treated with 0.1 to 1.0 mg CYC/mL. LDH release was significantly greater, however, in slices from the CYC-sensitive B mice. The highest toxicity occurred in response to 1.0 mg CYC/mL at 8 hr, where 63.9 ± 3.7% of LDH was released from B slices and only 45.8 ± 2.6% was released from the resistant C slices ($P < 0.05$ compared with controls and for comparison between strains). We also determined the effect of 3-AB on the toxicity of CYC. Co-incubation in 2.5 mM 3-AB was protective to lung slices from both strains (Figs. 1 and 2, panels B). In B slices, LDH release was reduced from 63.9 ± 3.7% to 44.7 ± 2.4% ($P < 0.05$) after 8 hr in 1.0 mg CYC/mL. Similarly, 3-AB reduced LDH release from 45.8 ± 2.6 to

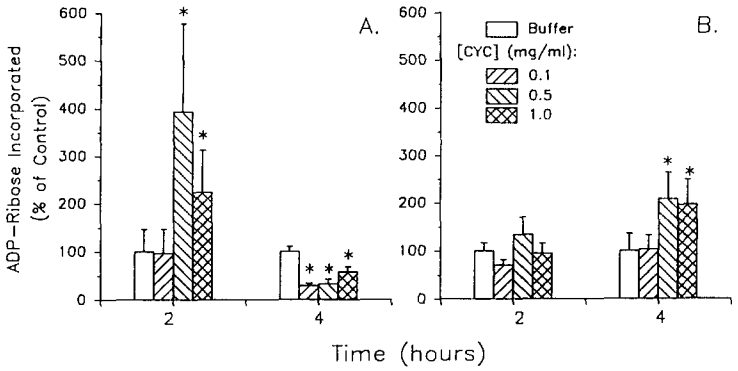

Fig. 3. PAP activity in nuclei isolated from BALB/cN mouse lung slices that were incubated with CYC in the absence (A) and presence (B) of 2.5 mM 3-AB. Activity is presented as percent of control (means ± SEM, N = 3). Control activities (fmol ADP-ribose incorporated/min/mg protein, means ± SEM) at 2 and 4 hr, respectively, were: panel A, 30.7 ± 7.8 and 57.9 ± 4.3; panel B, 33.3 ± 4.8 and 32.2 ± 8.1. Key: (*) $P < 0.05$ for comparison with buffer control.

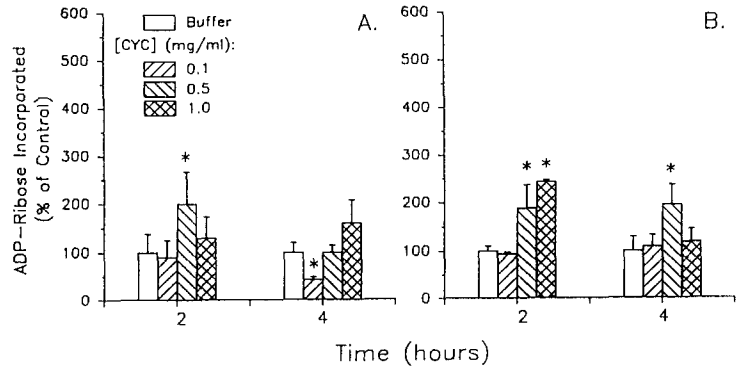

Fig. 4. PAP activity in nuclei isolated from C57Bl/6N mouse lung slices that were incubated with CYC in the absence (A) and presence (B) of 2.5 mM 3-AB. Data are presented as percent control (means ± SEM, N = 3). Control activities (fmol ADP-ribose incorporated/min/mg protein, means ± SEM) at 2 and 4 hr, respectively, were: panel A, 49.4 ± 13.1 and 47.9 ± 6.8; panel B, 26.3 ± 4.8 and 83.9 ± 17.3. Key: (*) $P < 0.05$ for comparison with buffer control.

28.1 ± 2.0% ($P < 0.05$) at the same concentration and time in C slices.

PAP activity. Nuclear PAP was activated 2- and 4-fold after treatment of B lung slices with 1.0 and 0.5 mg CYC/mL for 2 hr, respectively. This was followed by reduced activity at 4 hr (Fig. 3A). This biphasic pattern is typical for activation of PAP by DNA strand breakage. PAP activation was delayed and reduced when 3-AB was included in the incubation of slices with CYC (Fig. 3B). Incubation of C slices with 0.5 mg CYC/mL for 2 hr resulted in a 2-fold activation of nuclear PAP (Fig. 4A). Interestingly, in the presence of 3-AB, PAP was still activated by 0.5 and 1.0 mg CYC/mL at 2 hr. Activity remained elevated at 4 hr in nuclei from C slices treated with 0.5 mg CYC/mL and 3-AB (Fig. 4B). Thus, activation of PAP preceded LDH release and was greater in sensitive B lung slices than in C slices.

NAD. CYC caused a depletion of 0.7 to 1.9 nmol NAD/mg protein in the sensitive B lung slices within 2 hr, which continued through a 4-hr incubation (Fig. 5A). The lowest concentration of CYC, 0.1 mg/mL, decreased the NAD level only at 4 hr. In resistant C lung slices, NAD was decreased only at 4 hr with 1.0 mg CYC/mL (Fig. 6A).

NAD depletion by 0.1 mg CYC/mL at 4 hr in B slices was prevented by 3-AB (Fig. 5B). NAD depletion (decreases of 1.0 to 2.7 nmol NAD/mg protein) caused by the higher CYC concentrations was not prevented. In C slices, loss of NAD was also seen in slices treated with 3-AB and 1.0 mg CYC/mL at 2 and 4 hr. 3-AB caused NAD to increase at 6 hr after incubation in 0.1 and 0.5 mg CYC/mL (Fig. 6B).

NADP. CYC increased NADP levels in B and C lung slices (Figs. 7 and 8). NADP did not increase until 4 hr, and was similar in slices from both strains (0.2 to 0.35 nmol/mg protein). 3-AB enhanced the

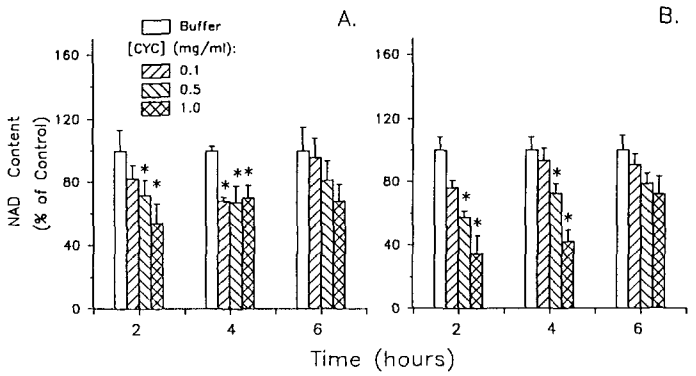

Fig. 5. Effect of CYC on the NAD content of BALB/cN mouse lung slices in the absence (A) and presence (B) of 2.5 mM 3-AB. Data are presented as percent of control (means ± SEM, N = 3). Control values (nmol/mg protein, means ± SEM, N = 3) at 2, 4 and 6 hr, respectively, were: panel A, 3.9 ± 0.43, 5.0 ± 0.51 and 2.2 ± 0.27; panel B, 2.8 ± 0.20, 4.6 ± 0.32 and 3.0 ± 0.23. Key:(*) $P < 0.05$ for comparison with buffer control.

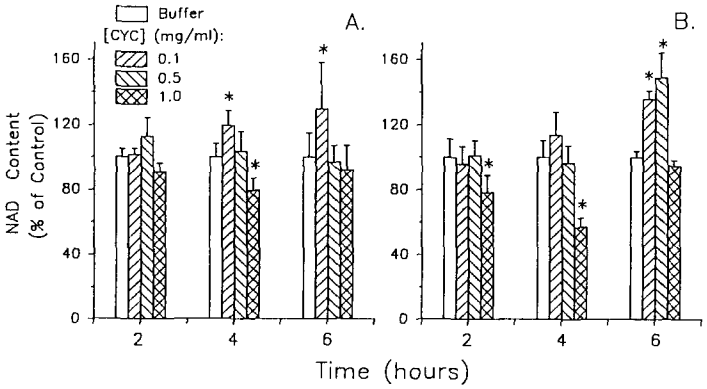

Fig. 6. Effect of CYC on the NAD content of C57Bl/6N mouse lung slices in the absence (A) and presence (B) of 2.5 mM 3-AB. Data are presented as percent of control (means ± SEM, N = 3). Control values (nmol/mg protein, means ± SEM, N = 3) at 2, 4 and 6 hr, respectively, were: panel A, 4.9 ± 0.21, 5.4 ± 0.30 and 3.1 ± 0.37; panel B, 4.5 ± 0.42, 4.3 ± 0.36 and 3.9 ± 0.15. Key: (*) $P < 0.05$ for comparison with buffer control.

increases in NADP, particularly in response to 0.5 and 1.0 mg CYC/mL. For example, NADP was now increased at 2 hr with these concentrations of CYC in B slices, and later increases ranged from 0.1 to 0.7 nmol/mg protein. However, 3-AB inhibited the increase in NADP caused by 0.1 mg/mL CYC at 4 hr in lung slices from both murine strains.

ATP. CYC caused an increase in ATP in sensitive B slices (Fig. 9A). 3-AB enhanced ATP levels at 4 hr of incubation with 1.0 mg CYC/mL (Fig. 9B). CYC only caused ATP depletion in the resistant C slices. ATP was decreased by approximately 25% at 4 hr of incubation with 0.5 mg/mL and after a 6-hr treatment with all concentrations (Fig. 10A). Co-treatment with 3-AB tended to increase ATP in CYC-treated C lung slices, where ATP levels were elevated after 2 and 6 hr of treatment with 1.0 mg CYC/mL (Fig. 10B).

DISCUSSION

Bioactivation of CYC is necessary for its toxic and chemotherapeutic actions [16]. This was investigated in mice by Kanekal *et al.* [32], who injected ICR and C57Bl/6 mice with a fibrogenic dose of radiolabeled CYC (200 mg/kg, i.p.). Pharmacokinetic calculations, using a two-phase elimination model and their data, indicate average blood concentrations (mg CYC/mL blood, averaged over specific time intervals from injection) ranging from 0.24 over 2 hr to 0.085 for an 8-hr period, a range embraced in the present study. Kanekal *et al.* [32] found that the blood levels, integrated over time, and the covalently bound radiolabeled metabolites of CYC in lung tissue correlated with the relative sensitivity of ICR mice compared with C57Bl/6 mice. Thus, lungs of resistant mice may be subject

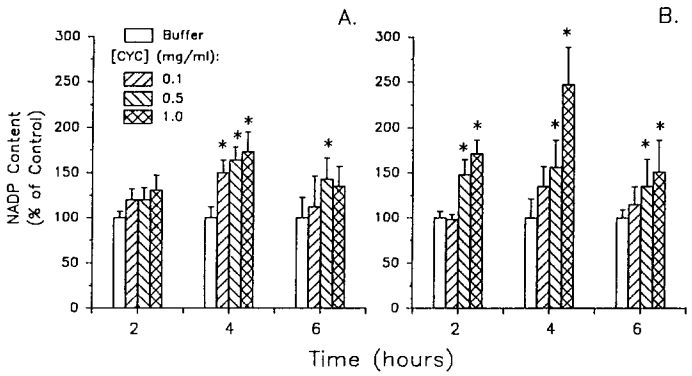

Fig. 7. Effect of CYC on the NADP content of BALB/cN mouse lung slices in the absence (A) and presence (B) of 2.5 mM 3-AB. Data are presented as percent of control (means ± SEM, N = 3). Control values (nmol/mg protein, means ± SEM, N = 3) at 2, 4 and 6 hr, respectively, were: panel A, 0.47 ± 0.04, 0.47 ± 0.04 and 0.53 ± 0.08; panel B, 0.37 ± 0.02, 0.46 ± 0.07 and 0.78 ± 0.05. Key: (*) $P < 0.05$ for comparison with buffer control.

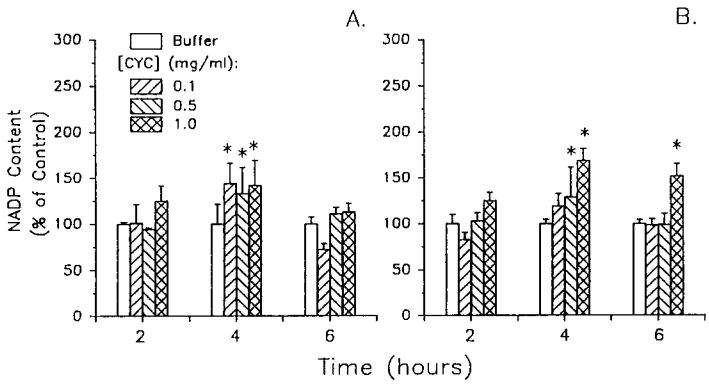

Fig. 8. Effect of CYC on the NADP content of C57B/6N mouse lung slices in the absence (A) and presence (B) of 2.5 mM 3-AB. Data are presented as percent of control (means ± SEM, N = 3). Control values (nmol/mg protein, means ± SEM, N = 3) at 2, 4 and 6 hr, respectively, were: panel A, 0.49 ± 0.007, 0.70 ± 0.10 and 0.62 ± 0.03; panel B, 0.48 ± 0.03, 0.72 ± 0.02 and 0.76 ± 0.02. Key: (*) $P < 0.05$ for comparison with buffer control.

to lower exposure or binding of activated metabolites *in vivo*. The authors, however, did not rule out parameters of intrinsic susceptibility of lung from these data. Other studies by the same group indicate that lungs activate CYC by arachidonic acid-stimulated cooxidation [10, 12].

Acute injury to endothelial and epithelial pneumocytes is a common event in drug-induced fibrosis *in vivo* [1]. In the present study, we found that CYC is toxic to isolated lung slices. Furthermore, murine strain sensitivity to acute cell injury, measured by the release of LDH from lung slices, correlates with the sensitivity of B mice and the resistance of C mice to CYC-induced pulmonary fibrosis *in vivo* (Figs. 1 and 2) [7]. Similar findings were obtained with lung slices exposed to BLM: C mice are sensitive to BLM and B mice are resistant [4, 9]. As suggested by Kanekal *et al.* with respect to C57Bl/6 and ICR mice [32], our results are consistent with the hypothesis that the extent of initial lung injury governs murine strain variation in response to BLM and CYC. Strain variation in response to CYC may be due to several factors. Activation or inactivation of CYC may vary between the strains, or the biochemical lesions caused by CYC, or repair, may be different in the two strains.

Lung slices were protected by 3-AB, suggesting a role for DNA damage and PAP in CYC-induced lung toxicity. In contrast to BLM, 2.5 mM 3-AB did not completely prevent CYC-induced LDH release (Figs. 1 and 2). PAP activation was greater in nuclei from CYC-treated B slices than C slices (Figs. 3 and 4) and activation was delayed, but incompletely blocked, in nuclei taken from B slices treated with CYC and 3-AB (Fig. 3). PAP activation was less in C slices than in B slices and was not blocked by 3-AB (Fig. 4). 3-AB could inhibit LDH release by additional means. Bioactivation of CYC may be altered, for example. This has been seen regarding the toxicity of 1,2-dibromo-3-chloropropane, where

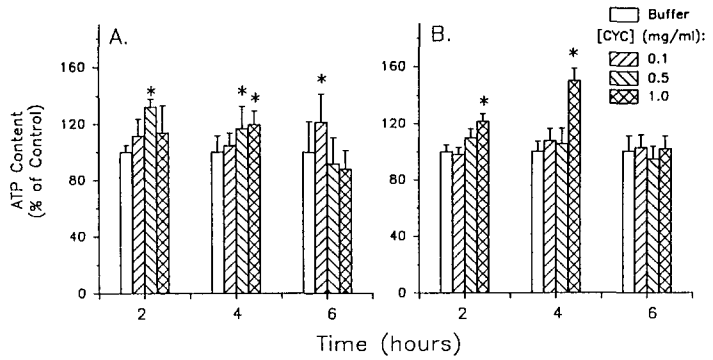

Fig. 9. Effect of CYC on the ATP content of BALB/cN mouse lung slices in the absence (A) and presence (B) of 2.5 mM 3-AB. Data are presented as percent of control (means ± SEM, N = 3). Control values (nmol/mg protein, means ± SEM, N = 3) at 2, 4 and 6 hr, respectively, were: panel A, 5.0 ± 0.20, 6.2 ± 0.59 and 4.8 ± 0.86; panel B, 5.3 ± 0.21, 6.7 ± 0.39 and 6.7 ± 0.60. Key: (*) $P < 0.05$ for comparison with buffer control.

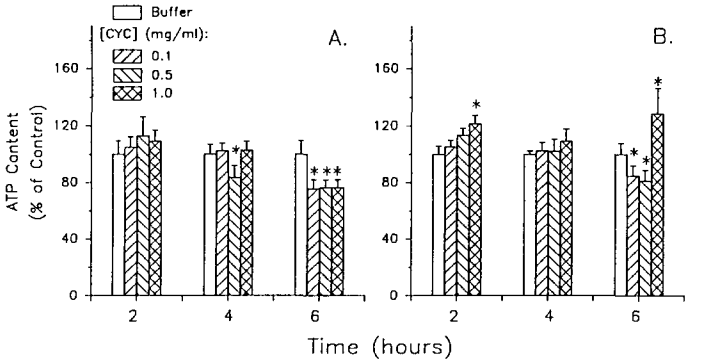

Fig. 10. Effect of CYC on the ATP content of C57B/6N mouse lung slices in the absence (A) and presence (B) of 2.5 mM 3-AB. Data are presented as percent of control (means ± SEM, N = 3). Control values (nmol/mg protein, means ± SEM, N = 3) at 2, 4 and 6 hr, respectively, were: panel A, 4.8 ± 0.38, 6.5 ± 0.20 and 6.1 ± 0.48; panel B, 4.2 ± 0.20, 7.3 ± 0.15 and 6.4 ± 0.41. Key: (*) $P < 0.05$ for comparison with buffer control.

3-AB reduced the level of metabolites covalently bound to rat tissues *in vivo* [33]. 3-AB may have radical scavenging properties, which also offer protection [22].

CYC caused depletion of NAD in the sensitive B lung slices (Fig. 5). This result is similar to our findings with BLM, which causes NAD depletion in BLM-sensitive C lung slices [19]. NAD depletion did not occur in the resistant C slices treated with CYC alone, although LDH was released (Fig. 6). The effect of 3-AB on NAD was complex. Loss of NAD was prevented by 3-AB in B slices at 4 hr of incubation with the lowest concentration of CYC, and NAD levels were increased by 3-AB at 6 hr in C slices. NAD depletion due to high concentrations of CYC, however, was not prevented by 3-AB in B slices (Fig. 5). NAD may be metabolized by mono(ADP-ribosylation) and glycohydrolase reactions in addition to PAP [34, 35]. Higher concentrations of benzamides (>2.5 mM) are required to block mono(ADP-ribosylation) and NAD glycohydrolase activity [34]. The effect of CYC on these reactions in lung is not known. Thus, the balance of PAP activity, mono(ADP-ribosylation) and NAD glycohydrolase activity in the presence of 2.5 mM 3-AB may complicate the final effect of the inhibitor on NAD levels. In addition, PAP activation is typically transient [9], as was seen in B slices (Fig. 3). Both self-inactivation and specific proteolysis of PAP yield proteins that continue to metabolize NAD, but that do not polymerize ADP-ribose as effectively as native PAP (i.e. an increase in NAD glycohydrolase activity may occur) [36, 37]. Thus, the *in vitro* assay of PAP measures enzyme activation, but may not indicate the magnitude of cellular NAD consumption by this metabolic path. Incomplete inhibition of activated PAP, its modified forms, or incomplete prevention of enzyme activation may explain partial protection of lung slices by 3-AB. The inability of 3-AB to block NAD depletion

completely may limit protection from CYC-induced injury. Alternatively, cells may not be injured by loss of NAD *per se*, placing emphasis on the role of PAP and its targets in toxicity. In any case, CYC-induced NAD depletion was a clear indicator of strain sensitivity.

NADP can be synthesized at the expense of NAD. This process is 3-AB-insensitive in quinone- or *t*-butyl hydroperoxide-treated hepatocytes, where NAD is decreased and NADP is elevated [21, 22]. In V79 cells, however, benzoquinone and naphthoquinone actually increase NAD and NADP levels simultaneously [38]. Activation of NAD phosphorylation may be cell type- and agent-specific. In our study, NADP increased after, or in the absence of, NAD depletion (see Figs. 5A–8A). In B slices, NAD decreases were approximately four times the increases in NADP (in nmol/mg protein) in all cases. Increases in NADP could be buffered by reduction to NADPH. Oxidative stress, due to peroxides, paraquat and toxic concentrations of quinones, causes NADPH depletion, presumably by oxidation [21, 22, 39]. These findings suggest that CYC-induced NAD depletion is not completely due to phosphorylation. Additional measurements of NAD and NADP metabolites are required to confirm this suggestion. Increased NADP, nevertheless, is consistent with induction of oxidative stress by CYC in lung [14].

CYC did not have a large effect on ATP levels, although significant reductions occurred late in the CYC-resistant C slices (Figs. 9 and 10). We previously found that BLM also had little effect on ATP levels in murine lung slices [19]. Our results are also consistent with observations by other investigators, indicating that ATP depletion does not necessarily result from NAD depletion [20, 40].

In conclusion, murine strain variation in CYC-induced pulmonary fibrosis correlated with LDH release in isolated lung slices directly exposed to CYC. 3-AB partially protected lung slices from both murine strains. PAP activation and NAD depletion preceded LDH release in sensitive B lung slices. PAP activation in B lung slices was reduced by 3-AB. Increased NADP may not account for all CYC-induced NAD depletion, but further experiments are necessary to be certain. The increases in NADP, which were similar in slices from both strains, are consistent with oxidative stress. CYC may cause injury by more than one mechanism, perhaps involving PAP activation and oxidative stress. Although cell injury by CYC *in vivo* is influenced by extrapulmonary factors, the results suggest that intrinsic pulmonary factors could contribute to the severity of CYC-induced fibrosis.

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REFERENCES

1. Adamson IY, Drug-induced pulmonary fibrosis. *Environ Health Perspect* **55**: 25–36, 1984.
2. Cooper JA, Jr, White DA and Matthay RA, Drug-induced pulmonary disease. Part 1: Cytotoxic drugs. *Am Rev Respir Dis* **133**: 321–340, 1986.
3. Gould VE and Miller J, Sclerosing alveolitis induced by cyclophosphamide: Ultrastructural observations on alveolar injury and repair. *Am J Pathol* **81**: 513–530, 1975.
4. Harrison JH and Lazo JS, High dose continuous infusion of bleomycin in mice: A new model for drug-induced pulmonary fibrosis. *J Pharmacol Exp Ther* **243**: 1185–1194, 1987.
5. Harrison JH and Lazo JS, Plasma and pulmonary pharmacokinetics of bleomycin in murine strains that are sensitive and resistant to bleomycin-induced pulmonary fibrosis. *J Pharmacol Exp Ther* **247**: 1052–1058, 1988.
6. Hoyt DG and Lazo JS, Alterations in pulmonary mRNA encoding procollagens, fibronectin and transforming growth factor-β precede bleomycin-induced pulmonary fibrosis in mice. *J Pharmacol Exp Ther* **246**: 765–771, 1988.
7. Hoyt DG and Lazo JS, Early increases in pulmonary mRNA encoding procollagens and transforming growth factor-β in mice sensitive to cyclophosphamide-induced pulmonary fibrosis. *J Pharmacol Exp Ther* **249**: 38–43, 1989.
8. Hoyt DG and Lazo JS, Bleomycin and cyclophosphamide increase pulmonary type IV procollagen mRNA in mice. *Am J Physiol* **259**: L47–L52, 1990.
9. Hoyt DG and Lazo JS, Murine strain differences in acute lung injury and activation of poly (ADP-ribose) polymerase by *in vitro* exposure of lung slices to bleomycin. *Am J Respir Cell Mol Biol* **7**: 645–651, 1992.
10. Smith RD and Kehrer JP, Cooxidation of cyclophosphamide as an alternative pathway for its bioactivation and lung toxicity. *Cancer Res* **51**: 542–548, 1991.
11. Kachel DL and Martin WJ, Cyclophosphamide-induced lung toxicity: Mechanism of endothelial cell injury. *J Pharmacol Exp Ther* **268**: 42–46, 1994.
12. Kanekal S and Kehrer JP, Evidence for peroxidase-mediated metabolism of cyclophosphamide. *Drug Metab Dispos* **21**: 37–42, 1993.
13. Patel JM, Block ER and Hood CI, Biochemical indices of cyclophosphamide-induced lung toxicity. *Toxicol Appl Pharmacol* **76**: 128–138, 1984.
14. Patel JM, Metabolism and pulmonary toxicity of cyclophosphamide. *Pharmacol Ther* **47**: 137–146, 1990.
15. Schraufstatter IU, Hinshaw DB, Hyslop PA, Spragg RG and Cochrane CG, Oxidant injury of cells. DNA strand-breaks activate polyadenosine diphosphate-ribose polymerase and lead to depletion of nicotinamide adenine dinucleotide. *J Clin Invest* **77**: 1312–1320, 1986.
16. Sladek NE, Oxazaphosphorines. In: *Metabolism and Action of Anti-cancer Drugs* (Eds. Powis G and Prough RA), pp. 48–90. Taylor & Francis, Philadelphia, 1987.
17. Benjamin RC and Gill DM, Poly(ADP-ribose) synthesis *in vitro* programmed by damaged DNA: A comparison of DNA molecules containing different types of strand breaks. *J Biol Chem* **255**: 10502–10508, 1980.
18. Ueda K and Hayaishi O, ADP-ribosylation. *Annu Rev Biochem* **54**: 73–100, 1985.
19. Hoyt DG and Lazo JS, NAD depletion after *in vitro* exposure of murine lung slices to bleomycin. *Biochem Pharmacol* **46**: 1819–1824, 1993.
20. Stubberfield CR and Cohen GM, NAD^+ depletion and cytotoxicity in isolated hepatocytes. *Biochem Pharmacol* **37**: 3967–3974, 1988.
21. Stubberfield CR and Cohen GM, Interconversion of NAD(H) to NADP(H). A cellular response to quinone-

induced oxidative stress in isolated hepatocytes. *Biochem Pharmacol* **38**: 2631–2637, 1989.
22. Yamamoto K and Farber JL, Metabolism of pyridine nucleotides in cultured rat hepatocytes intoxicated with *tert*-butyl hydroperoxide. *Biochem Pharmacol* **43**: 1119–1126, 1992.
23. Berger NA, Oxidant-induced cytotoxicity: A challenge for metabolic modulation. *Am J Respir Cell Mol Biol* **4**: 1–3, 1991.
24. Carson DA, Seto S, Wasson DB and Carrera CJ, DNA strand breaks, NAD metabolism, and programmed cell death. *Exp Cell Res* **164**: 273–281, 1986.
25. Hardwick SJ, Adam A, Smith LL and Cohen GM, Potentiation of cell specific toxicity of paraquat by 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU): Implications for the heterogeneous distribution of glutathione (GSH) in rat lung. *Biochem Pharmacol* **39**: 581–589, 1990.
26. Moldeus P, Hogberg J and Orrenius S, Isolation and use of liver cells. *Methods Enzymol* **52**: 60–71, 1978.
27. Bradford MM, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**: 248–254, 1976.
28. Maniatis T, Fritsch EF and Sambrook J, *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1982.
29. Jones DP, Determination of pyridine dinucleotides in cell extracts by high-performance liquid chromatography. *J Chromatogr* **225**: 446–449, 1981.
30. Pogolotti AL and Santi DV, High-pressure liquid chromatography–ultraviolet analysis of intracellular nucleotides. *Anal Biochem* **126**: 335–345, 1982.
31. Snedecor GW and Cochran WG, *Statistical Methods*, 7th Edn. Iowa State University Press, Ames, IA, 1980.
32. Kanekal S, Fraiser L and Kehrer JP, Pharmacokinetics, metabolic activation, and lung toxicity of cyclophosphamide in C57/BL6 and ICR mice. *Toxicol Appl Pharmacol* **114**: 1–8, 1992.
33. Holme JA, Sonderlund EJ, Lag M, Brunborg G and Dybing E, Prevention of 1,2-dibromo-3-chloropropane (DBCP-induced kidney necrosis and testicular atrophy by 3-aminobenzamide. *Toxicol Appl Pharmacol* **110**: 118–128, 1991.
34. Jacobson MK, Rankin PL and Jacobson EL, An approach to the selective targeting of NAD consuming reactions in cultured mammalian cells. In: *ADP-Ribose Transfer Reactions: Mechanisms and Biological Significance* (Eds. Jacobson MK, and Jacobson EL). pp. 361–365. Springer, New York, 1989.
35. Kim H, Jacobson EL and Jacobson MK, Synthesis and degradation of cyclic ADP-ribose by NAD glycohydrolases. *Science* **261**: 1330–1333, 1993.
36. Desmarais Y, Menard L, Lagueux J and Poirier GG, Enzymological properties of poly(ADP-ribose)-polymerase: Characterization of automodification sites and NADase activity. *Biochim Biophys Acta* **1078**: 179–186, 1991.
37. Kaufmann SH, Desnoyers S, Ottaviano Y, Davidson NE and Poirier GG, Specific proteolytic cleavage of poly(ADP-ribose) polymerase: An early marker of chemotherapy-induced apoptosis. *Cancer Res* **53**: 3976–3985, 1993.
38. Ludewig G, Dogra S and Glatt H, Genotoxicity of 1,4-benzoquinone and 1,4-naphthoquinone in relation to effects on glutathione and NAD(P)H levels in V79 cells. *Environ Health Perspect* **82**: 223–228, 1989.
39. Witschi H, Kacew S, Hirai KI and Cote MG, *In vivo* oxidation of reduced nicotinamide-adenine dinucleotide phosphate by paraquat and diquat in rat lung. *Chem Biol Interact* **19**: 143–160, 1977.
40. Andreoli SP, Mechanisms of endothelial cell ATP depletion after oxidant injury. *Pediatr Res* **25**: 97–101, 1989.